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NUCLEAR PHENOMENA ASSOCIATED WITH HETEROTHALLISM AND HOMOTHALLISM IN THE ASCOMYCETE NEUROSPORA

BY

B. O. DODGE

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NUCLEAR PHENOMENA ASSOCIATED WITH HETEROTHALLISM AND HOMOTHALLISM IN THE ASCOMYCETE NEUROSPORA[1]

By B. O. Dodge

Pathologist, Office of Fruit Diseases, Bureau of Plant Industry, United States Department of Agriculture

INTRODUCTION

A new genus of ascomycetes with four species has been recently described by Shear and Dodge (*18*)[2]. The culture work reported in that paper established the connection between the conidial and the ascocarpic stages. It also proved that two of the species, *Neurospora sitophila* and *Neurospora crassa*, are heterothallic. A preliminary study of the nuclei in spore formation in *Neurospora tetrasperma*, which is ordinarily homothallic, suggested that by a proper selection of spores on the basis of their size, monosporous mycelia could be obtained which would produce only conidia when grown separately, but which when properly mated would also develop ascocarps. The present paper discusses the nuclear behavior which accounts for the fact that heterothallic strains may be segregated out of a species which is commonly homothallic.

The ascus of *Neurospora tetrasperma* usually develops only four spores, and each spore contains two nuclei at its origin. The method by which two or more of the eight nuclei in an ascus cooperate in the delimitation of an ascospore is described for the first time.

MATERIAL AND METHODS

Material for cytological work on *Neurospora tetrasperma* was obtained from cultures on corn-meal agar in test tubes and Petri dishes. If conidia or fragments of mycelium are sowed on corn-meal agar, perithecia will begin to appear about the fifth day, so that material for fixing may be had from cultures which are from 5 to 10 days old, depending on the temperature of the culture room.

Small pieces of agar bearing ascocarps were fixed in Flemming's weaker solution. Sections were cut 5 microns thick and stained with the triple stain. The perithecia usually form on the surface of the agar, so that orientation of the fruit bodies in cutting is not difficult. Not infrequently the perithecia develop below the surface of the agar. Such material is not as satisfactory for this work. The perithecia of *Neurospora crassa* do not fix as well as do those of *N. sitophila* and *N. tetrasperma*. Only those results obtained by a study of the last species are given in this paper.

[1] Received for publication Apr. 19, 1927; issued October, 1927.
[2] Reference is made by number (italic) to "Literature cited," p. 304.

NUCLEAR BEHAVIOR IN THE ASCUS OF NEUROSPORA TETRASPERMA

The ascus crosier appears to be formed in the usual fashion and later the two nuclei in the young ascus fuse. In this stage it is usually seen that the cytoplasm of the ascus has been differentiated into two regions. The central part, more finely granular, is to become the spore plasm. The epiplasm in the upper and lower ends of the ascus contains large vacuoles which are somewhat elongated.

After nuclear fusion has taken place and the young ascus has elongated, the development of a thickening at the apex of the ascus goes on until it can be recognized clearly as a red ring when stained with safranin. In fixed material the cytoplasm at both ends of the ascus seems inclined to shrink more than the central portion, perhaps because it is more vacuolar. The fusion nucleus is often surrounded by several so-called extranuclear bodies which stain deeply (pl. 1, A).

The spindle of the first division lies along the long axis of the ascus (pl. 1, B). In no case has a transverse spindle been found at this stage although the ascus is rather broad and there would be sufficient space for a transverse spindle.

After the first division is completed the reorganized daughter nuclei, moving apart, come to rest in various positions. They may be widely separated, one lying in the upper, the other in the lower part of the spore plasm. In that case the spindles of the second division will appear to be nearly longitudinal (pl. 1, D). This material gives an abundance of the two-nucleate stage, but the chromatin does not differentiate well in staining. The chromosomes in the metaphase stages of the first and second divisions are very distinct so that they might be counted without great difficulty. If the daughter nuclei had come to rest in the position shown in Plate 1, C, the two spindles would then be oriented in some such way as are those shown in Plate 1, E and F. At E the remains of the old

EXPLANATORY LEGEND FOR PLATE 1[1]

A.—Primary nucleus with extranuclear bodies. Cytoplasm differentiated into the central spore plasm, and epiplasm at each end with large vacuoles. Shrinkage during fixation at the upper and lower ends.

B.—Metaphase of the first division. The spindle is parallel to the long axis of the ascus. The pointed ends of the spindle take the safranin stain readily.

C.—One type of the two-nucleate stage where the nuclei are not oriented on the central longitudinal axis of the ascus; large vacuoles in either end of the ascus.

D.—Second division in metaphase stages. The spindles are longitudinal, one somewhat above the other but at a different focus.

E.—Another type of division in which the spindles lie oblique to the walls of the ascus. The remains of the old nuclear membrane somewhat collapsed, show distinctly on both of the spindles. Very little cytoplasm in either end of the ascus; spore plasm rather dense and finely granular.

F.—Late telophase stage of the second division. Spindles oblique, one somewhat above and parallel to the other. The polar chromatin masses connected by filamentous structures which take the stain rather heavily. Remains of the parent nuclear material seen at the center of the upper spindle.

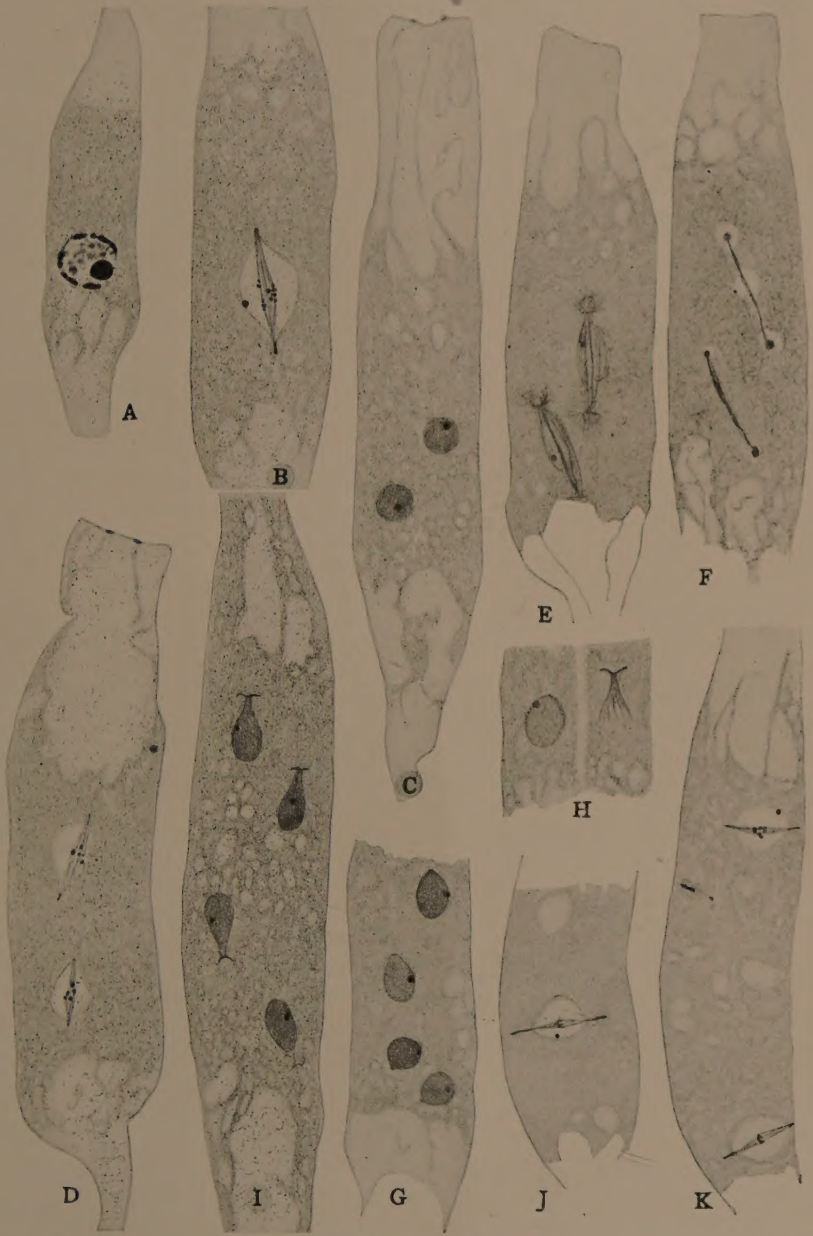
G.—Four-nucleate stage, the pair of nuclei lying at a higher focus, more heavily shaded. Such a distribution of the nuclei may well result from divisions similar to those shown in Figures E and F. The two upper nuclei are somewhat pear shaped. The relationship of the nuclei can be determined by the location of the central bodies; compare with I.

H.—Two views of the same nucleus somewhat more enlarged than in the other figures. At the left and seen at a higher focus, the nucleus appears nearly spherical or without particular distortion. At the right, part of the same nucleus, seen at a lower focus, now shows the beak-like cap with forked appendages.

I.—Four-nucleate stage in resting condition. Nucleoles distinct. Each nucleus is pear shaped or crowned by a beak-like cap of fibers at the end of which is a forked appendage. The beaks of the sister nuclei extend in opposite directions. The spore plasm at this stage is divided into two parts by a series of central vacuoles.

J and K.—Two sections of the same ascus during metaphase stages of the third division. J should have been mounted so that the nucleus would have been at about the same level as the lower nucleus in K. At the upper end of the ascus in K can be seen one complete spindle and the tip end of a second, the remainder of which appeared in the next section and was not drawn.

[1] The writer is indebted to Lulu O. Gaiser for assistance in drawing a number of the figures included in the plates.



(For explanatory legend see p. 290)

(For explanatory legend see p. 291)

nuclear membranes and the fibrous material connecting the sister polar masses are still visible. Slightly later stages are shown in F. The daughter nuclei in each pair are beginning to reorganize while the darkly staining connecting strands are still very prominent.

In about half of the figures of the second division catalogued the spindles present the appearance of conjugate division. They lie more or less parallel to each other and oblique to the long axis of the ascus. Divisions in the ascus have heretofore been regarded merely as simultaneous. Conjugate divisions are devices to insure nonsister nuclei being brought together in a common cytoplasm. With such a method of division as is shown in Plate 1, E and F, each end of the ascus would surely contain a pair of nonsister nuclei. If such an arrangement serves a useful purpose, and it would if segregation of the sex factors occurs in the first division, how can such a contingency be provided for in case the dividing nuclei are located as shown in Plate 1, D? Fully 50 per cent of the division stages at this time actually show these spindles rather widely separated. This would suggest that after the second division there would of necessity be a pair of sister nuclei in each end of the spore plasm all in one row. The writer has never seen such an orientation in *Neurospora tetrasperma*. Since they do not lie on the same axis of the ascus, a shifting of position of the reorganizing nuclei, still connected by fibers, must take place, so that a pair of nonsister nuclei will come to lie in each end of the ascus. This is similar to the shifting of pairs of nuclei in the promycelium described by Dodge and Gaiser (*5*) for *Caeoma nitens*, except in a reverse order and with the opposite effect.

It will be shown later in the discussion that perhaps nothing in the cytology of the ascus is more important in its bearing on the experimental results obtained from growing ascospores and crossing different species of Neurospora, than is a knowledge of the genetic relationship of the two nuclei which one sees in each end of the ascus after the second division. Practically any section of a perithecium in which the asci are developing vigorously will show such figures as are given in Plate 1, I. Because of the beautiful symmetry presented in such cases there can be no doubt that the nuclei whose beaks extend in opposite directions are sister nuclei.

Each of the four nuclei shown in Plate 1, G, is slightly elongated. A central body is clearly evident at the point of greatest elongation of each nucleus—that is, on the side toward the top of the figure for the two upper nuclei and on the side toward the bottom of the figure for the two lower ones. The nuclei that are more heavily shaded

EXPLANATORY LEGEND FOR PLATE 2

A to C.—Three consecutive sections of the same ascus. The first section, A, shows a large part of one spindle and a part of one spike-like appendage of another. The rest of these nuclei appear at the lower end of the ascus, shown in B. The chromatin material is bunched at each end of the spindles and spike-like prolongations somewhat curved, extend out to the plasm membrane. The two nuclei shown in the upper end of the ascus, B and C, are members of a pair dividing conjugately, one almost directly under the other but more obliquely placed.

D.—Slightly older or late telophase stage. The daughter nuclei nearly reorganized and showing the curved spiked extensions now pressing against the plasma membrane on each side.

E and F.—Two adjacent sections of the same ascus showing the eight nuclei at the beginning of spore formation. The nuclei clearly in pairs, symmetrically placed, two on one side and two on the other in each end of the ascus. The appendages are now strongly curved and from these structures are proceeding very delicate astral rays. The spore plasm of the ascus is divided into two regions by large vacuoles arranged across the ascus at about the middle.

G.—Stage in spore formation in one end of an ascus somewhat later than the stages shown in E and F. Each spore will contain a pair of nonsister nuclei from the beginning.

were at a higher focus. They are undoubtedly sister nuclei. The best evidence for this assumption is that the central bodies are symmetrically located on opposite sides of the nuclei. Viewed at right angles to the plane of the section shown in Plate 1, G, such a disposition of the four nuclei could well have followed from an orientation of the spindles like that shown in E.

The writer has described the nuclei at this stage as pear shaped or as having long beaks (pl. 1, I). When, however, one studies such sections as are illustrated in Plate 1, H, or those of more delicately stained preparations, the beak sometimes appears to be made up of a cone of fine fibers capping the nucleus, which perhaps is only slightly elongated. In spite of this evidence the writer is not convinced that such figures represent early stages in the reorganizing of the daughter nuclei.

Another reason why Plate 1, I, is assumed to represent a stage long after the second division, is that the spore plasm is now very definitely divided into two sections by a series of vacuoles extending somewhat obliquely across the ascus. These vacuoles enlarge and run together more and more after the third division (pl. 2, E and F.) The organization of the fine granular material at the poles (pl. 1, E) suggests slightly the origin of the forked appendages, yet at the poles of the spindle in F there is not the slightest indication of such structures. Plate 1, F, clearly represents a later stage than E.

A study of slightly later and more critical stages may show that the antlerlike structures, central bodies(?), attached to the chromosomes by fibres, simply break in two and move straight out to the walls of the ascus on opposite sides, giving metaphase stages like those shown in Plate 1, J and K.

In the third division the spindles are transverse—that is, perpendicular to the ascus walls. In the metaphases of all three divisions one sees at each end of the spindles a sharply pointed mass of deeply staining substances, a part of which is undoubtedly the centrosome, although astral rays do not show at all prominently at any stage in this material.

Usually not all of the four spindles of the third division lie in one plane. The section may show a longitudinal view of the upper spindle, while the lower spindle appears only in a polar view. The sections for the illustrations for Plate 1, J and K, and Plate 2, A and C, were chosen particularly because, showing longitudinal and not polar views of the spindles, they give the best idea of the relationship of the eight nuclei resulting from this set of divisions.

In the telophase stages the now curved and more densely staining end structures appear to be attached to the ascus walls, the chromatin bodies being bunched together in two or three masses (pl. 2, A, B, and C) and the remains of the old spindle still stretching across the ascus. A still later stage is shown in Plate 2, D, in which the reorganization of the daughter nuclei is about completed.

The next stage shows pairs of small nuclei side by side, pressing against the ascus wall. Symmetrically placed on the opposite side of the ascus, is another pair of similar nuclei (pl. 2, E and F). Seldom are all four nuclei seen in one section. Serial sections, however, invariably show the nuclei which were missed in the preceding section.

The beaks of the nuclei now become further prolonged and curved sharply at the ends like umbrella handles. Astral rays are very

delicate in these species so that the details in spore delimitation are not adequately brought out. There can be no doubt, however, as to the nature of the process or of the fact that adjacent nonsister nuclei cooperate in cutting out the spores. In the early stages it is sometimes impossible to tell whether one very large spore or two of normal size will be delimited in one end of an ascus, especially in cases where four nuclei lie in nearly the same plane (pl. 2, G). If the plasma membrane and vacuoles take part in the process, it is only in a secondary way. Large vacuoles often appear both within and outside the spore opposite the nuclei (pl. 3, A and B).

There must be much twisting, turning, and slipping of the spores just as soon as they are fully cut out; otherwise they would not be uniseriate at maturity, and such sections as are shown in Plate 3, A, in which the two nuclei in each of the four spores all point in the same direction, would not be found. Three such cases were observed in this material. The nuclei in adjacent spores usually extend in opposite directions (pl. 3, B, above). The curved tip ends of the beaks must be fairly definite structures (pl. 3, C). With delimitation complete, the body of the nucleus becomes detached from the curved end (pl. 3, D) and moves down to the center of the spore (pl. 3, E). After the four spores have enlarged somewhat and become more or less elliptical, their nuclei divide simultaneously. The spindles in a spore usually are not in the same plane. Parts of two adjacent sections of the same ascus are shown in Plate 3, F and G. The spindles of the spore at *a* and *a'* are at right angles. The mature normal spore will have four nuclei (pl. 3, H).

SIGNIFICANCE OF ABNORMAL SPORES

The most interesting phase of spore formation is brought out in Plate 3, B, where the formation of four spores, each with a single nucleus, is going on in one end of an ascus while two spores with two nuclei each are being cut out in the other end. The genetic relationship of the nuclei in the uninucleate spores can be told by the position of the beaks, which extend in opposite directions when the nuclei are sisters. The two adjacent spores at the right of Plate 3, B, as well as the two at the left would have been of opposite sex; but it is problematical where they would finally lie in the mature ascus. In a narrow ascus, such as is found in *Neurospora crassa* and *N. sitophila*, the two spores developed from sister nuclei are always adjacent in the mature ascus. Further work is necessary to learn the details of the processes by which more than two nuclei cooperate in spore formation in *N. tetrasperma*. As noted previously, an ascus of this species normally contains four spores (fig. 1, A). If one crushes four or five perithecia in which asci are maturing, he will probably find one or two asci with an abnormal number of spores (fig. 1, B to E); most rarely of all, an ascus may contain only one giant spore. If the nuclei of one sex be indicated in black and those of the other sex in white, Figure 1, F and G, will represent the way in which the nuclei cooperate in cutting out spores with two nuclei, and also the way in which they sometimes fail to cooperate, with the result that small uninucleate, unisexual spores are developed. Figure 1, H and I, show how either four or eight nuclei could act together in cutting out large spores. This point has not been followed up.

Shear and Dodge (*18*) found that monosporous mycelia from small spores develop large numbers of sclerotia or bodies which resemble aborted perithecia. No ascospores are developed in such structures. By properly mating these cultures, normal perithecia are formed with asci which have commonly only four spores. The larger, normal spores, have two nuclei when first delimited and thus contain nuclei of both sexes.

These conditions are in an entirely different category from those described by Burger (*3*). Cunninghamella is heterothallic. Burger reports isolation of monosporous mycelia which he says may be called hermaphroditic. Work with these aberrant mycelia was entirely discontinued so that the cultures were lost and this work has not been repeated or confirmed. What little is known of the cytology of the Mucoraceae would suggest that Burger may well have been correct in his conclusions. The condition described might depend on where and how completely segregation takes place, and the chances for the accidental inclusion of nuclei of both sexes in the same spore at some critical point following the germination of the zygospore. Such an explanation, however, is discounted by Burger, who is inclined to entertain the idea of compatibility, although he has no explanation to offer for the condition which he describes.

The writer refers to monosporous mycelia of the heterothallic species as haplonts and uses the terms "unisexual," "bisexual," "sex," and "sexuality" in this paper without knowing, in fact, whether or not the mycelia of the species of Neurospora produce functional oogonia and antheridia. Both reciprocal haplonts produce coiled structures which enter into the development of the perithecium. Further study will no doubt demonstrate that these primordia are morphologically and functionally different. Work on crossing of the haplonts of different species, to be reported later, shows that the descendants of nuclei from both haplonts must finally fuse in the ascus.

Knowing that the existence of "psuedo-heterothallism" in *Neurospora tetrasperma* is based on the accidental inclusion of only one of the two sexually different nuclei which are normally contained in each spore at its origin, a way is suggested by which one may be able to obtain homothallic strains from heterothallic species, such as *N. crassa* and *N. sitophila*. The former species particularly may,

EXPLANATORY LEGEND FOR PLATE 3

A.—Section of an ascus showing four spores just after they have been completely delimited. Each spore contains a pair of nuclei close together. In this case the spores have readjusted their positions so the beaks in the four pairs all extend in the same general direction. The epiplasm is considerably shrunken from fixation and the spores have not as yet assumed their final elliptical shape. Very fine astral rays are still visible proceeding from the curved projections of the nuclei.

B.—Ascus showing the basis for the development of homothallic and heterothallic spores in the same ascus. Each of the two spores in the upper end of the ascus contains two nuclei of opposite sex. Each of the four spores in the lower end of the ascus contains only a single nucleus. Since the nuclear beaks of the two spores at the right extend in the same direction, they are probably of opposite sexes. Each spore of the pair at the left is also unisexual. The spores with one nucleus are much smaller than those containing two.

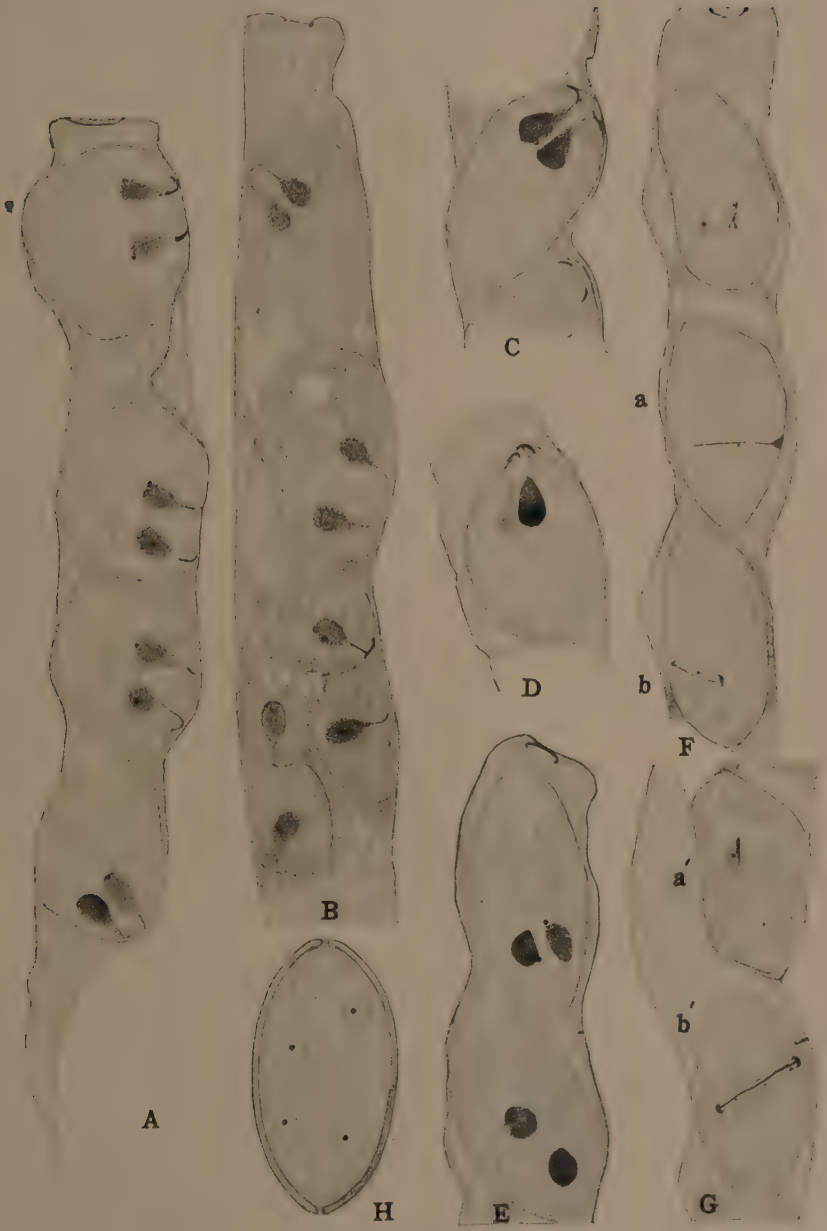
C.—Later stage in spore formation. The nuclei about to break away from the tip ends of the curved beaks. In the lower of the two spores, the appendages which were cut off in sectioning are indicated at the right.

D.—Still later stage in spore formation. The nuclei have been completely detached from the appendages, which are still visible at the upper end of the spore.

E.—The daughter nuclei have become nearly rounded up and the spores no longer show the remains of the hooked nuclear beaks.

F and G.—Parts of two sections of the same ascus showing dividing nuclei in telophase stage. At a and a′ are shown two sections of the same spore, and at b and b′ two sections of the next lower spore. Two nuclei in each of the four spores of this ascus were all in the same stage of division

H.—A mature spore after reorganization of the four daughter nuclei and the thickening of the spore wall; a germ pore is visible at each end



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on rare occasions, develop an ascospore which is much too large for a spore with only one nucleus at its origin (fig. 1) and can not be accounted for as simply a matter of competition for the nourishment in the spore plasm. Should such a spore contain two or more nuclei at its origin (fig. 1, G, H) and have been delimited in the same way as are the normal spores of *N. tetrasperma*, then, on germination, a homothallic mycelium will be developed. Clearly heterothallism and homothallism in the species of Neurospora are not absolutely fixed specific characters, although the sexual nature of an individual haplont is definitely determined by the time the spore is cut out.

The occurrence of spores of different sizes in the ascus of *Bulgaria inquinans* has long been a matter for speculation. Moreau (*14*) finds that some spores are large and others are small for two very different reasons. All of the nuclei do not necessarily divide simultaneously so that one may sometimes find resting nuclei of both the second and third generations in the ascus at the same time. A nucleus of the second generation will be much larger than one of the third. Spores formed at once and including nuclei of the third division, this author believes, having the first chance at the nourishment in the spore plasm, would become larger than the other spores formed in the same ascus later. Regardless of the size of the spore, each one contains only one nucleus at its origin. When two of the original eight nuclei are included in the same spore, as Wolf (*20*) found in *Podospora anserina*, the spore will be much larger than one with only a single nucleus. Such forms as *P. anserina* and *Bulgaria polymorpha* should prove extremely interesting. Has the former a heterothallic sister species? May not the smaller uninucleate spores of the latter be unisexual and the larger binucleate spores be bisexual as they are in *Neurospora tetrasperma?* Faull (*6*) figures a two nucleate spore of Neotiella, and Fraser (*9*) shows a spore of *Humaria rutilans* containing five nuclei at its origin. These authors considered such spores simply as abnormal and of no consequence. The writer has pointed out above the significance of the inclusion of different numbers of nuclei in spores, particularly in cases where heterothallism must be taken into account.

FIG. 1.—Diagram of asci with various numbers of spores at maturity and method of spore delimitation. A, normal number of spores is four, very rarely only one giant spore (E) is formed; F, small uninucleate, unisexual spores; G, bisexual, binucleate spores; H, bisexual four-nucleate spore; I, eight-nucleate spore in which all of the nuclei resulting from the third division of the fusion nucleus cooperate in cutting out the spore. H and I theoretical

HETEROTHALLISM NOT DIOECISM

Perhaps 50 per cent of the red algae are heterothallic. In the filamentous and thalloid green and brown algae the male and female

gametes of certain species are borne on different plants. The same is true for liverworts, mosses, and ferns; and, of course, all seed plants are heterothallic. Among the fungi, certain species of water molds have long been known to be heterothallic. Thanks particularly to Thaxter, one order of ascomycetes alone, the Laboulbeniales, now presents at least a dozen genera with some hundred species which are heterothallic. In all of the groups mentioned, it is readily demonstrated that some gametophytes are male and others are female, although the cytological details of nuclear behavior may be entirely unknown. With such evidence at hand, it is strange that the existence of heterothallism among our higher ascomycetes should have been so long unknown.

Blakeslee (*2*), when he first proposed the use of the terms, stated that "heterothallic" and "homothallic" correspond to "dioecious" and "monoecious" in the flowering plants. In all his subsequent writings this author shows that originally he must have intended the illustration merely as an analogy. Allen (*1*) working on the genetics of Sphaerocarpos has emphasized the importance of keeping in mind the difference between male and female plants of the liverworts, mosses, etc., on the one hand, and staminate and pistillate individuals of the flowering plants on the other. Pollen tubes are male haplonts, embryo sacs are female haplonts. Recently Sharp (*17*) in discussing the factorial interpretation of sex determination rightly insists that all flowering plants are heterothallic. Only a comparatively small number are dioecious. Less confusion might result if, for example, such genera as Amorphomyces and Herpomyces were referred to as heterothallic instead of as dioecious. The terms now have entirely different meanings.

The Mucoraceae apparently do not afford the best material for cytological study. No one has been able so far to find any connection between nuclear behavior and the segregation of the factors determining sexual reproduction in heterothallic forms.

In the case of certain higher basidiomycetes it is now known just what the nuclei do, step by step, from the time the spores germinate until the new generation of spores is formed on the basidium. No more complete story of nuclear behavior is known in the plant kingdom, but there is nothing in the cytology which serves as a basis for predicting which species of Coprinus, for example, will be heterothallic and which ones homothallic.

Melanospora destruens (*18*) resembles *Neurospora sitophila* in several morphological features. It is not yet clear why the former should be homothallic and the latter heterothallic. Within the genus Neurospora, however, the factors which determine certain types of sexual reaction are clearly segregated before, instead of after, spore formation, so that a study of nuclear behavior in the ascus enables one to see why one species is heterothallic while another is homothallic.

SEGREGATION OF SEX FACTORS

In Pyronema the antheridia are developed on dichotomously branched hyphae which are quite distinct from those that build the oogonia. Monosporous mycelia of this species are said (*4*) to produce ascocarps in culture. Each ascospore is at first uninucleate. The segregation of the sexes must therefore take place a long time after

spore germination. A number of ascomycetes outside of the Laboulbeniales develop sex organs which are morphologically and functionally of two different sorts. Since the two spores in an ascus of *Phyllactinia corylea* are formed from sister nuclei (*11*) the chances, one might think, for this species to be heterothallic are small. Yet if the factors for sex differentiation are segregated out in the third division, as may well be the case, spores of two different sexes will then be formed in the same ascus. The production of perithecia by certain species of the Erysiphaceae is very erratic. When culture work on such forms is carried out with monosporous haplonts, no doubt some of these species, such as the mildew on raspberries, will likely be found to be heterothallic.

Thaxter's highly interesting accounts of the development of various species of the Laboulbeniales show that in the heterothallic ("dioecious") forms the spores are of two sorts, usually somewhat different in size. The male spore is frequently somewhat smaller than the female. They are discharged from the asci in pairs and become attached to the host side by side. The sex of each spore has been determined absolutely by the time it has been delimited. In this connection Thaxter remarks (*19, v. 13, p. 222*): "The cytological phenomena * * * which lead up to the sexual differentiation and pairing in the ascus of these male and female spores, is likely to prove of unusual interest." It is a curious fact that in cases where the spores of the hermaphroditic species are frequently discharged in pairs and become attached to the host side by side, only one of the spores of the pair develops into a mature plant (*19, v. 14*). The other spore degenerates without germination.

Thaxter (*19, v. 12, p. 217–218*) further says: "It has been previously mentioned that in a majority of forms [hermaphroditic species] the antheridial appendage is developed from the terminal cell of the germinating spore. The female organs, however, are always formed from the products of the division of the basal cell, never in any case from the terminal cell, where this is present. Although the products of the division of the terminal cell are invariably sterile or male, it is not true, as might be supposed, that the basal cell or its derivatives have any inherent female character, since in many cases both normal and abnormal antheridia and antheridial branches may arise below the point of insertion of the female organ, or even, in exceptional cases, replace it entirely."

This may be the correct interpretation. It may also be claimed that the facts cited go to show conclusively that usually the terminal cell is in fact inherently male and the basal cell female, and that segregation of the sexes takes place during the fourth nuclear division after nuclear fusion, that is, during the first nuclear division after spores have been cut out. Occasionally, however, such a sex segregation is not consummated at this time and antheridial appendages develop from the cell which, normally or abnormally, gives rise to the carpogenic structures. This could very well be due to a failure to lay down the septum until after a second nuclear division in the spore, and the inclusion of one of the extra male nuclei in the basal cell. If the development of the male structures from the terminal cell and the female structures from the basal cell could be reversed

by inverting the spore or in any other way, this would prove, of course, that there is nothing inherent sexually in either segment of the spore.

Thaxter has been unable to determine whether or not the asci of the genus Herpomyces are eight-spored. "If this were actually the case," he says, "it would involve the curious phenomenon of absolute sex differentiation in the last mitosis, which would not necessarily occur in a four-spored ascus" (*19, v. 13, p. 222*).

It is to be regretted that the only cytological work so far published on the Laboulbeniales has been upon two species in which the antheridial structures are said to be unknown, so that sexual reproduction as such would clearly be out of the question. Faull (*8*) finds that in *Laboulbenia chaetophora* the spindles of the first and second mitoses are longitudinal. In the four-nucleate stage the nuclei are in pairs, two in the upper part of the ascus and two in the lower; again in the third division the spindles are longitudinal. After reorganization, four nuclei move to the upper part of the ascus and disintegrate. The four which develop at the lower ends of the spindles take a central position. Faull says further (*7*): "The functional nuclei, in *Laboulbenia chaetophora* at least, are the lower ones in the spindles of the last mitosis. There are some reasons for believing that the same is true of Amorphomyces and Dicichomyces, in which case sexual differentiation of the spores might be determined in the second division."

Assuming that nuclear behavior during the processes leading up to the delimitation of the ascospores should be the same for the heterothallic genera as that given by Faull for the two parthenogenetic species which he studied, is it necessary to assume also that the segregation of the sex factors must occur in the second division? For practically all the species of this group where fecundation is an essential factor for the full development of the ascus, all that would be necessary to make a species heterothallic would be to move the point of segregation back from the fourth to the third nuclear division. Evidently many of the species commonly homothallic have provided for just such an emergency by discharging the spores in pairs.

The features in the cytology of the ascus discussed by previous authors and which have a particular bearing on segregation of the sex factors and other work covered in this paper are: (1) The position and direction taken by the spindles during each of the three nuclear divisions; (2) the location of the resting nuclei in the spore plasm and their relationship, one to the other, at different stages in the development of the ascus; and (3) the part taken by the nuclei during spore formation and the method of spore delimitation. It may be noted that on certain points there is a general agreement among previous writers.

The spindle in the first division of the primary nucleus usually lies parallel to the long axis of the ascus. Harper (*10*) and Claussen (*4*) have pointed out that in Pyronema this spindle may also be transverse. The writer has found that in *Ascobolus winteri* the first spindle is always transverse. One or two other exceptions have been reported.

After the first division has been completed, the two daughter nuclei separate and come to lie one above the other, either on the

median long axis of the ascus, or, just as frequently, on some line which is oblique to the ascus walls. Reports of finding the nuclei lying side by side as though reorganized at the ends of a transverse spindle have not been seen in the literature.

The two spindles of the second division are usually described as lying along the general direction of the ascus axis. In Pyronema, according to Claussen (*4*), and in Phyllactinia, according to Harper (*11*), one of the two spindles of the second division may be transverse. As a general rule when an ascus is long and narrow the four nuclei, following a second division, lie in a row on the long axis of the ascus. The two nuclei in each end of the axis of the ascus would then be sister nuclei. The disposition of the four nuclei must depend largely on how much of the cytoplasm of the axis is differentiated into spore plasm. Jolivette (*16*) says that if a transverse section of the ascus were made after the second division in *Geoglossum glabrum*, the four nuclei would be seen in the same plane. In a longitudinal section two nuclei are seen in one focus and two in the other. The spindles of the third division show no tendency to lie in the transverse axis of the asci.

There are a few other reports of the lack of uniformity in the disposition of the spindles in the third division. Faull (*7*, *8*) claims that all four of the spindles of the third division in Laboulbenia are longitudinal. In most other ascomycetes, however, they are reported to be more or less transverse. Komarnitzky (*12*) and Faull have both remarked on the possible connection or bearing which the genetic relationship of particular nuclei may have on "dioecism" in the Laboulbeniaceae. Their work will be considered further in the general discussion. In most ascomycetes division figures are so rarely found that one should not draw conclusions as to the significance of the orientation of the spindles in certain cases in which it is clear that the author has based his report on only one or two spindles which he was fortunate enough to find.

The first spindle in *Verpa bohemica* (*12*) is nearly longitudinal. The spore plasm is developed in the upper end of the ascus and all of the nuclear divisions occur in this end. Of the two spindles in the second division, the upper appears to be transverse, the lower somewhat oblique. The only spindle shown in the third division is nearly transversely placed. The evidence presented by Komarnitzky's figures is not conclusive as to the particular nuclei which are concerned in spore formation. Two spores are finally cut out and the other six nuclei degenerate. Komarnitzky realizes, however, the importance of knowing the exact orientation of the spindles during the three different divisions in the ascus.

If no switching or exchange of position of the nuclei takes place after their reconstruction, in other words, if one is justified in drawing conclusions as to the genetic relationship of particular nuclei from the position and orientation of the division figures, then in the "dioecious" forms of the Laboulbeniaceae, segregation of the sex factors may very well take place in any one of the three divisions with the same result, provided the nuclei take the positions and the spindles are oriented as Faull (*8*) figures for Laboulbenia. In all three divisions the spindles extend lengthwise of the ascus. Faull (*8*, *fig. 57*) shows that the four nuclei resulting from the second mitosis lie in pairs, two in one end of the ascus and two in the other, comparable

to what occurs in *Neurospora tetrasperma* (pl. 1, I). It will appear in due time, however, that one would be led far astray were he to presume to draw conclusions as to the time of segregation and the sexual nature of the eight spores in the heterothallic species *N. sitophila* from what he finds to be true of nuclear behavior in the homothallic form *N. tetrasperma*.

Full information regarding the early prophase stages as the four nuclei of *Neurospora tetrasperma* go into the third division, has not been obtained. This is a critical stage, not only because of its bearing on the question as to where segregation takes place, but also because further light is desirable as to the nature of the structures which always appear as forked appendages at the beaks of the nuclei at this time. If these horns represent merely lines of flow, or a massing due to fixation of astral rays, one would not expect to find such regularity in their form and position. They certainly appear more like definite organs, in the nature of monstrous central bodies or blepharoplasts.

Harper (*11*) shows that immediately after the first division the two nuclei in Phyllactinia are pear-shaped. He states that they always round up, however, before beginning the second division. The same thing may take place in the case of the four nucleate stage of Neurospora so that the stage shown in Plate 1, I, would precede that shown in Plate 1, G. There is much evidence against such a view, however. First, the two pairs of nuclei undergoing the third division are at opposite ends of the ascus and widely separated. There is practically no change of position after the hornlike appendages have developed. In the stage shown in Plate 1, G, the nuclei are not in the final position for the third division. The beaked nuclei in Plate 1, I, may not be fully mature or reorganized, yet they are certainly in a resting condition and must so endure for a long time. Such stages are very abundant in the material. If the nuclei dispose of the beaks and forked appendages before going into the third division, they must do so in a very short space of time. With the development of the spindle of the last division, each end of it is provided again with a curved spikelike structure which extends out to the plasma membrane. After the first division on the contrary the two nuclei round up and remain in this condition for some time.

Since the factors upon which sexual reproduction in the heterothallic species of Neurospora depends are lodged in two different spores and in two different nuclei of the same spore in homothallic species, it is essential to a proper understanding of the questions involved to determine at what time and in what way the segregation of the sex factors takes place.

Shear and Dodge (*18*) have reported that in *Neurospora crassa* four spores in an ascus are of one sex and four of the other. Nothing is said concerning the position in the ascus of each of the eight spores.

Students of heterothallism of the basidiomycetes, forced to explain some of the curious results of their culture work, are assuming that two pairs of factors, "sex factors," control the reactions leading to the formation of clamp connections. Newton (*15*) believes that in *Coprinus lagopus* segregation of one factor may take place in the

first division in the basidium and the segregation of the other factor in the second division. The writer has not been confronted with such complicated situations so far, yet the results obtained by crossing *Neurospora sitophila* with *N. tetrasperma* show the need of exact knowledge of nuclear behavior through the entire life cycle in both species as well as in the different hybrid generations. It will be shown in the following diagrams that nuclear behavior in the ascus of *N. tetrasperma* is such that normally totipotent or homothallic ascospores will be formed, regardless of whether segregation takes place in the first, second, or third division.

One may take as a type an ascus such as that of *Galactinia succosa* figured by Maire (*13*), which shows very diagrammatically the position of the nuclei and orientation of the spindles generally reported for long asci. If segregation of the sex factors takes place in the first division, then as indicated in the diagram (fig. 2) the four spores in the upper end of the ascus must be all alike, and all of one sex, and the four in the lower end must be of the other sex. If segregation of the sex factors takes place in the second division in a species the spores will alternate in pairs, the first two being of one sex and the next two of the opposite sex. With a knowledge of conditions in the ascus of *N. tetrasperma*, a brief study of nuclear behavior in the ascus of *N. sitophila* has convinced the writer that the spores of the latter species will so alternate in pairs. Should segregation take place in the third division, then the four spores in one end of the ascus will alternate as regards their sex. So it is clear that with no shifting of position of the nuclei, three different pictures will result, depending on the place of segregation.

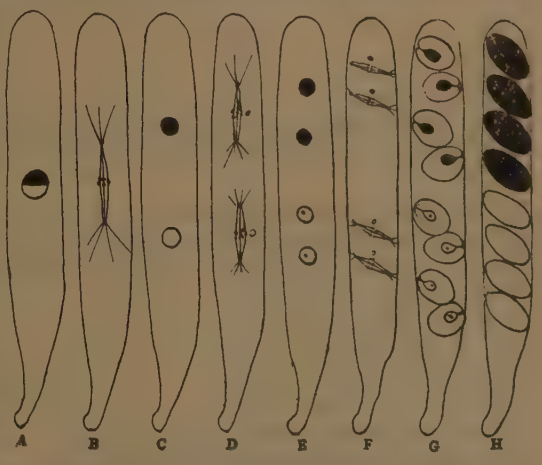

FIG. 2.—Diagram showing nuclear behavior and position of the four spores of each sex in a typical long ascus if segregation of the sex factors should occur in the first mitosis

In *Neurospora crassa* and *N. sitophila*, after the second divisions, the four nuclei lie in a row in the ascus. In no case has the writer found, as previously noted, such a condition in *N. tetrasperma*. The evidence so far discovered indicates that regardless of the position of the two nuclei during the second division in the last species such a shifting must take place as is necessary to bring the four nuclei into the formation shown in Figure 3, E, which is the one picture that seems to be very constant in this material (pl. 1, I).

Should segregation take place in the first mitosis in *Neurospora tetrasperma* the way in which each spore would receive at its origin one nucleus of each sex is indicated in Figure 3, A to H. The spindle of the first division is always longitudinal. The essential difference

between this type of nuclear behavior and that shown in Figure 2 is not in the number of nuclei in the young spore. In a number of ascomycetes with long asci it has been demonstrated, as can be done

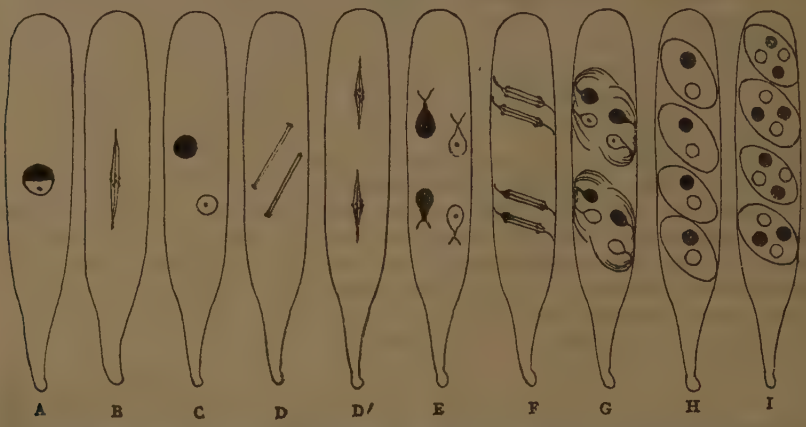

FIG. 3.—Diagram showing nuclear behavior and method of formation of bisexual spores in *Neurospora tetrasperma*. The position of the spindles in the second mitosis may be parallel to each other and oblique to the long axis of the ascus, D, or they may be longitudinal, one in each end of the ascus, D′

easily in case of *N. sitophila* and *N. crassa*, that the two nuclei in each end of the ascus are sisters (fig. 2, E). With segregation taking place in either the second or third division such an arrangement would accord with the experimental results obtained with *N. tetrasperma* should two adjacent nuclei be included in the same spore. Yet the evidence obtained by a study of the shape and the position of the four nuclei of this species after they have come to rest (fig. 3, E) indicates that here, as previously noted, a shifting of the nuclei must occur after divisions like those shown in Plate 2, D, or Figure 3, D′; otherwise fully 50 per cent of the asci at this stage would show four nuclei in a row down the center, which certainly is not the case. No such orientation has been found, although this would account for the occasional formation of large spores which are unisexual.

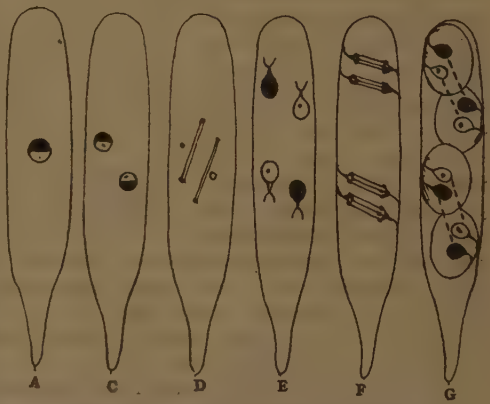

FIG. 4.—Diagram of nuclear condition leading to the development of bisexual spores in *Neurospora tetrasperma*, should segregation of the sex factors occur in the second mitoris, other conditions as in Figure 3

With segregation in the second division in *Neurospora tetrasperma*, the spores will all be bisexual (fig. 4, A to G) no matter in which of the two ways (fig. 3, D or fig. 3, D′) the spindles of the second mitosis are oriented. Occasionally in the case of this species, only one

nucleus is included in each of certain spores (pl. 3, B). With nuclear behavior otherwise the same, such spores would alternate in pairs in the ascus, two being of one sex and two of the other.

Figure 5, A to G, shows how segregation might take place in the third division without preventing the formation of bisexual spores. Perhaps 1 per cent of the monosporous mycelia from what was judged to be either normal spores, or spores which were oversized, failed to develop perithecia. One is forced to find an explanation for this apparent failure in such cases to include nuclei of both sexes in these spores. A section of an ascus which was taken to be abnormal showed what appears to be a pair of nuclei in the second division, with spindles transverse, one in the upper, the other in the lower end of the ascus. Such abnormal conditions are generally said to be of no particular significance, yet these cases may be the very ones to account for unusual conditions, such as the development of large unisexual spores with two or even more nuclei at their origin.

The sexual nature of each of the eight spores in the ascus of *Neurospora sitophila* has been determined culturally by Marguerite Wilcox who has assisted the writer in the preparation of slides for this cytological study. Her report on this work, together with a further account of the cytology of the ascus in this species, is forthcoming. With the cytology of the ascus known and the sexual nature of each of the spores in the ascus of *N. tetrasperma* and *N. sitophila* worked out culturally, the way is cleared for a better interpretation of the results being obtained by crossing reciprocally unisexual haplonts of these two species.

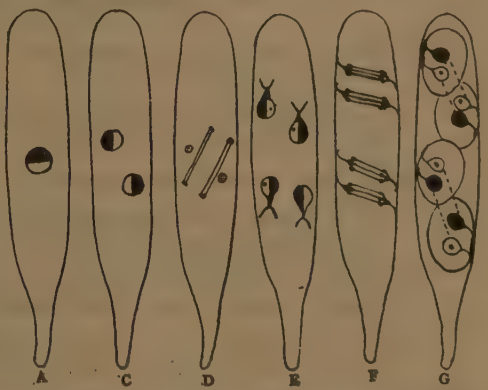

FIG. 5.—Diagram of nuclear condition in an ascus of *Neurospora tetrasperma*, showing the possibility of the formation of bisexual spores, should segregation of the sex factors occur in the third mitosis, other conditions as in Figure 3

SUMMARY

Neurospora tetrasperma, which is normally homothallic, develops asci with four bisexual spores, as contrasted with the heterothallic species, *N. sitophila*, in which the asci are eight spored, and each spore is unisexual. The cytological basis for this difference is reported.

In *Neurospora tetrasperma*, the spindle of the first mitosis is longitudinal; the two daughter nuclei separate and come to rest one somewhat above the other in the ascus.

With respect to the position and orientation of the spindles of the second division two types are described. In the first type the spindles may lie nearly parallel near the center of the ascus, usually somewhat oblique to the long axis, suggesting conjugate division. In the second type, the spindles are longitudinal, one in each end of the ascus.

Each of the four nuclei is pear shaped and the beak is capped by a forked appendage. The nuclei are arranged symmetrically, two nonsister nuclei in each end of the ascus.

The spindles of the third mitosis are nearly transverse. Normally two adjacent nonsister nuclei cooperate in the delimitation of each ascospore through the development of astral rays from their curved beaks. It is shown how, regardless of whether segregation takes place in the first, second, or third division, each spore will contain one nucleus of each sex.

Occasionally adjacent nuclei, for some reason, are not entirely compatible and fail to cooperate in the cutting out of a spore. Each nucleus then acts independently, and as a result uninucleate, unisexual spores, which are comparatively small, are delimited. Very rarely all eight of the nuclei in an ascus cooperate in the delimitation of a spore. Such an ascus will contain only a single giant spore at maturity.

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